

## **Echinacea and its alkylamides: Effects on the influenza A-induced secretion of cytokines, chemokines, and PGE<sub>2</sub> from RAW 264.7 macrophage-like cells**

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### **Abstract:**

The goal of this study was to determine whether extracts and isolated alkylamides from *Echinacea purpurea* would be useful for prevention of the inflammatory response that accompanies infections with H1N1 influenza A. Seventeen extracts and 4 alkylamides were tested for the ability to inhibit production of cytokines, chemokines, and PGE<sub>2</sub> from RAW 264.7 macrophage-like cells infected with the H1N1 influenza A strain PR/8/34. The alkylamides undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide, dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide, dodeca-2E,4E-dienoic acid isobutylamide, and undeca-2E-ene-8,10-diynoic acid isobutylamide suppressed production of TNF- $\alpha$  and PGE<sub>2</sub> from infected cells. Dodeca-2E,4E-dienoic acid isobutylamide was especially effective at inhibiting production of these mediators and also strongly inhibited production of G-CSF, CCL2/MCP-1, CCL3/MIP-1 $\alpha$  and CCL5/RANTES. In contrast, the ethanol extracts (75%), which were prepared from dormant roots of *E. purpurea* grown in different locations throughout North Carolina, displayed a range of effects from suppression to stimulation of mediator production. Precipitation of the extracts with ethanol removed the stimulatory activity, however, even after precipitation; many of the extracts did not display any suppressive activity. Analysis of the extracts revealed slight variations in concentration of alkylamides, caftaric acid, and cichoric acid, but the activity of the extracts did not strongly correlate with concentrations of these compounds. Our in vitro

experiments suggest that *E. purpurea* extracts have the potential for use in alleviating the symptoms and pathology associated with infections with influenza A; however, further study will be necessary to define procedures necessary to unmask the alkylamide activity in crude extracts.

**Keywords:** Echinacea | H1N1 influenza | Alkylamide | Isobutylamide | Plant extracts | Anti-inflammatory

## Article:

### 1. Introduction

According to figures reported in the Nutrition Business Journal, sales of *Echinacea* products in the US gross \$120 million annually <sup>[1]</sup>. *Echinacea* is also widely used outside the US, for example, physicians in Germany write over 3 million prescriptions for *Echinacea* products each year <sup>[2]</sup>. *Echinacea* preparations, primarily *Echinacea purpurea*, *Echinacea angustifolia*, and *E. pallida*, are employed for the treatment of upper respiratory infections. However, there is still a lack of agreement in the scientific community as to their effectiveness for this purpose, and clinical trials have yielded conflicting results <sup>[3], [4], [5] and [6]</sup>. Part of this confusion arises because *Echinacea* is typically used in the form of a crude extract or pill made from powdered plant material. Such preparations are inherently complex and variable in composition. While some of the *Echinacea* extracts used for therapeutic purposes are standardized to particular constituent levels, there is no consensus in the US dietary supplements industry as to which constituents should be used. This is not surprising given that there is still disagreement in the scientific community as to which constituents from *Echinacea* are responsible for its purported usefulness against upper respiratory infections, and how these constituents act. This lack of knowledge prevents effective quality control of *Echinacea* and limits the ability to conduct successful clinical trials.

*E. purpurea* is the most commonly used and studied species of *Echinacea*, and is the subject of the studies described herein. The types of *E. purpurea* preparations that are most often employed therapeutically include ethanol extracts (which typically contain approximately 60% ethanol) <sup>[7]</sup>, and aqueous extracts or “fresh pressed juice,” which are prepared without ethanol, but are typically preserved with a lower percentage of ethanol (in the range of 20%) <sup>[8]</sup>. The composition of complex *Echinacea* extracts varies depending on processing techniques and plant material used. It is generally assumed that ethanol extracts contain higher levels of alkylamides and phenolic compounds, while aqueous extracts are more likely to contain hydrophilic compounds such as polysaccharides and glycoproteins <sup>[9]</sup>.

The current general consensus in the field of *Echinacea* research is that *Echinacea* preparations can have either stimulatory or suppressive effects on innate immunity depending on the nature of the preparation used. *Echinacea* fresh pressed juice <sup>[10]</sup> as well as crude ground *Echinacea* root <sup>[11] and [12]</sup>, appears to enhance immunity by increasing production of

certain cytokines from macrophages and monocytes. It is often suggested that this stimulatory effect may aid the body in warding off infection<sup>[13]</sup>, and perhaps be helpful for preventing colds and flu. Polysaccharides<sup>[14], [15], [16] and [17]</sup> and/or lipopolysaccharides and lipoproteins<sup>[18] and [19]</sup> present in these *Echinacea* extracts may be responsible for their immunostimulatory activity. These compounds may come not just from the *Echinacea* plant itself, but from bacteria that are either introduced into the preparation as contamination or live in the plant as endophytes<sup>[18]</sup>.

There is a growing community of scientists that attributes the usefulness of ethanolic *Echinacea* preparations for treating infection to their ability to suppress the inflammatory response, thereby suppressing symptoms associated with the infection<sup>[20] and [21]</sup>. This activity is generally attributed to alkylamides and/or caffeic acid derivatives that are present at high levels in ethanolic extracts<sup>[13]</sup>. It is notable that such anti-inflammatory activity would constitute a “suppression” of innate immunity, as opposed to the immune enhancement that has typically been attributed to aqueous extracts of *Echinacea*. Sharma et al.<sup>[22]</sup> have shown that Echinforce®, a standardized ethanol extract prepared from fresh *E. purpurea* roots, can suppress cytokine and chemokine production from human blood monocytes stimulated by respiratory viruses. Furthermore, a number of studies with ethanolic *Echinacea* extracts or alkylamides from *Echinacea* preparations have shown that they can inhibit the LPS-induced production of TNF- $\alpha$ , IL-1, IL-6, and IL-8 cytokines and chemokines from human monocytes/macrophages and from macrophage-like cell lines<sup>[23], [24], [25] and [26]</sup>. The alkylamides appear to mediate these effects through both cannabinoid receptor (CB)-dependent and CB-independent mechanisms<sup>[24] and [26]</sup>.

In this investigation, we focused on the virus influenza A. Over 30,000 Americans die annually from infections with influenza A, and new treatments are clearly necessary. Many of the symptoms and pathology associated with this disease, including; fever<sup>[27]</sup>, anorexia<sup>[28]</sup>, nasal discharge<sup>[29]</sup>, and pain<sup>[30]</sup> are dependent on the production of a “storm” of cytokines, chemokines, and inflammatory lipids. Given the previous studies suggesting anti-inflammatory activity of alkylamides from *Echinacea*, it is reasonable to hypothesize that the purported relief provided by *Echinacea* for influenza may stem from the ability of these compounds to suppress production of inflammatory mediators from virus infected macrophages. The goal of our studies was to evaluate the effects of alkylamides and alkylamide-containing *E. purpurea* extracts from diverse geographical locations on the production of inflammatory cytokines, chemokines, and lipids from influenza A-infected RAW 264.7 macrophages. Our overall objectives in conducting these experiments were to gain insight into the mode of anti-inflammatory activity of alkylamides from *E. purpurea* and to determine whether these compounds serve as useful biomarkers for the in vitro anti-inflammatory activity of complex *E. purpurea* extracts.

## 2. Methods

### 2.1. Plant material and standards

Dormant *E. purpurea* roots were harvested from 17 cultivation sites across North Carolina between February and May 2008. Sites ranged from commercial farm fields to private gardens. Sites were selected to provide a wide representation of soils, climates, altitudes, and general growing conditions. In many cases, original seed sources were unknown. Plant ages varied from approximately 2 to 10 years at time of harvest. Roots were dug, washed, and dried at 38 °C under positive air flow. Voucher specimens for each harvest location were pressed, dried, and deposited in the University of North Carolina Herbarium (CB# 3280, Coker Hall, University of North Carolina, Chapel Hill, NC 27599-3280). Accession numbers correspond to the sample numbers listed in Table 1 as follows: sample 1 — NCU585854, sample 2 — NCU585842, sample 3 — NCU585843, sample 4 — NCU585858, sample 5 — NCU585848, sample 6 — NCU585859, sample 7 — NCU585851, sample 8 — NCU585849, sample 9 — NCU585860, sample 10 — NCU585852, sample 11 — NCU585846, sample 12 — NCU585853, sample 13 — NCU585856, sample 14 — NCU585857, sample 15 — NCU585844, sample 16 — NCU585845, and sample 17 — NCU585847.

**Table 1.** Concentrations of the most abundant alkylamide, 11a/b (dodecatetraenoic acid isobutylamide) and caffeic acid derivatives (caftaric acid and cichoric acid) present in the ethanol precipitated extracts (85% ethanol) *after dilution into the assay well*. The same dilution was employed for all extracts, and original extract concentrations of extracts (prior to dilution into sample wells) are 172 times higher. For example, the extract concentration for dodecatetraenoic acid isobutylamide in sample 01 prior to dilution was 3.8 mM (0.9 mg/mL). Sample 7 displayed the most pronounced anti-inflammatory activity ( Fig. 6).

Sample #	Concentration Alkylamide 11a/b		Concentration Caftaric acid		Concentration Cichoric acid	
	μM	μg/mL	μM	μg/mL	μM	μg/mL
01	22.0	5.4	2.9	0.16	2.9	0.24
02	11.0	2.7	4.1	0.22	4.6	0.38
03	16.8	4.2	2.9	0.16	3.5	0.28
04	16.2	4.0	3.5	0.19	3.5	0.28
05	13.3	3.3	3.5	0.19	1.7	0.14
06	9.3	2.3	7.5	0.41	12.2	1.00
07	22.0	5.4	3.5	0.19	2.9	0.24

08	11.6	2.9	5.2	0.28	4.1	0.33
09	10.4	2.6	2.9	0.16	2.9	0.24
10	9.3	2.3	4.1	0.22	2.3	0.19
11	5.2	1.3	2.9	0.16	2.3	0.19
12	4.6	1.1	2.3	0.12	2.3	0.19
13	14.5	3.6	2.9	0.16	2.9	0.24
14	11.0	2.7	4.6	0.25	7.0	0.57
15	20.3	5.0	3.5	0.19	7.0	0.57
16	10.4	2.6	7.5	0.41	8.7	0.71
17	17.4	4.3	3.5	0.19	7.0	0.57

Standards for alkylamides 4 (undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide), 11a/b (dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide) and 15 (dodeca-2E,4E-dienoic acid isobutylamide) were obtained from Gaia Herbs (Brevard, NC, USA) and were identified based on retention time comparison and MS–MS fragmentation according to published literature <sup>[31], [32] and [33]</sup>. Standards for alkylamide 16 (undeca-2E-ene-8,10-diynoic acid isobutylamide) and the caffeic acid derivatives caferic acid and cichoric acid were purchased from Chromadex (Santa Ana, CA, USA) with certificate of analysis indicating correct identification. Stock solutions were prepared from the standards in ethanol to produce final concentrations of 0.1, 10, 50, 100, and 500  $\mu$ M.

## 2.2. Extraction

Samples of dried *E. purpurea* roots were extracted within three weeks of drying. The whole dried roots were cut, and pulverized into a fine powder using a blender. The powder was macerated for seven days in 75% ethanol (Pharmaco-AAPER, Shelbyville, KY, USA) at a ratio of 1:5 (g plant material:mL solvent). The resulting extract was pressed using a hydraulic press and filter-sterilized through a 0.2  $\mu$ M membrane under vacuum in a laminar flow hood using sterile technique. All extracts were stored in sterile polypropylene bottles at 2–8 °C until the time of analysis. The limulus amebocyte lysate (LAL) assay (Lonza, Basel, Switzerland) was performed on these extracts according to standard methods. Test results indicated all extracts to be free of endotoxin (lipopolysaccharide) with a sensitivity of 0.25 EU/mL. Extracts were tested for their effects on cultured macrophage-like cells in their original form, and also following precipitation in ethanol. To conduct ethanol precipitation, extracts were combined with ethanol

(95%) at a ratio of 1 mL extract:1 mL ethanol (final 85% ethanol). The resulting mixtures were centrifuged, and the supernatant was retained and will hitherto be referred to as “precipitated extracts.”

### 2.3. Analysis of extracts

Reversed phase HPLC coupled with electrospray ionization mass spectrometry (ESI-MS) was employed to analyze the precipitated extracts, in accordance with previously published methods<sup>[32]</sup>. An HP1100 HPLC system (Agilent, Palo Alto, CA, USA) with a narrow bore C18 column (50 mm × 2.1 mm, 110 Å pore size, 3 µm particle size, Grace, Deerfield, IL, USA) was used. A precolumn filter (0.5 µm) (MacMod analytical, Chadds Ford, PA, USA) was employed in series with the column to filter out particulates. Binary gradient elution was employed at a flow rate of 0.2 mL/min with solvent A consisting of 1% acetic acid in nanopure water and solvent B consisting of HPLC grade acetonitrile (Pharmaco-AAPER, Shelbyville, KY, USA). Analyses were conducted separately for alkylamides and caffeic acid derivatives. For alkylamides, the solvent composition was as follows: t = 0–4 min, a constant composition of A–B (90:10, v/v); for t = 4–15 min, a linear gradient from A–B (90:10, v/v) to A–B (60:40, v/v); for 15–30 min, a linear gradient from A–B (60:40, v/v) to A–B (40:60, v/v); for t = 30–35 min, a constant composition of A–B (90:10, v/v). For caffeic acid derivatives, the solvent composition was: t = 0–39 min, a linear gradient from A–B (90:10, v/v) to A–B (50:50, v/v); for t = 39–43 min, a constant composition of A–B (90:10, v/v). An ion trap mass spectrometer with electrospray ionization (ESI) source (LCQ Advantage, Thermo Fisher, San Jose, CA, USA) was used. The mass spectrometer was operated over a scan range of 50–2000. Capillary, spray, and tube lens offset voltages were 3 V, 4.5 kV, and –60 V, respectively. Analyses of alkylamides were conducted in the positive ion mode while analyses of caffeic acid derivatives were conducted in the negative ion mode.

### 2.4. Cells and media

Murine RAW 264.7 macrophage-like cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco's Modified Eagle's (DME) Medium with 4 mM l-glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate with 10% fetal calf serum (FCS). All media and supplements were obtained from Sigma-Aldrich (St. Louis, MO, USA). FCS was obtained from Atlanta Biologicals (Atlanta, GA, USA). For production of cell culture supernatants,  $1.5 \times 10^5$  cells/well were plated in 24 well tissue culture plates in 1 mL culture media. Cells were treated with extracts by diluting directly into cell media (6.7 µL of extracts prior to precipitation or 5.8 µL of precipitated extracts into a 1 mL well volume, for a final ethanol percentage of 0.5% in both cases). These amounts were selected to allow testing at a relevant concentration of alkylamide (see Table 1), while still keeping ethanol below a toxic level. Following treatment, supernatants were collected, centrifuged for 2 min at 8000 rpm to remove debris, aliquoted, and stored at –80 °C.

## 2.5. Influenza A virus propagation

Influenza A PR/8/34 (VR-1469) was purchased from the American Type Culture Collection (Manassas, VA, USA) and propagated in MDCK Cells (ATCC CCL-34). T-75 flasks of cells at 90% confluency were inoculated with 0.01 MOI of virus in 2 mL of virus growth medium (VGM) made up of DMEM containing 0.2% bovine serum albumin (BSA), 25 mM Hepes buffer, 100 U/mL Penicillin, 100 µg/mL Streptomycin, and 2 µg/mL TPCK-treated Trypsin (LS003740, Worthington-Biochem, Lakewood, NJ, USA). Viral supernatants were harvested at 36 to 48 h, centrifuged to remove cellular debris, and supplemented with BSA to a final concentration of 0.5%. Aliquots were frozen and stored at  $-80^{\circ}\text{C}$ . Titers of influenza A virus were determined by plaque assay using MDCK cells. Briefly, 200 µL of serially diluted virus in VGM was inoculated onto confluent MDCK cells in 24-well plates. After a 30 min absorption period, 0.8 mL of overlay was added (0.6% Tragacanth in VGM). After 48 h of incubation, the overlay was removed, the cells washed with cold PBS, fixed with cold acetone:methanol (1:1), and stained with crystal violet.

## 2.6. ELISAs and arrays

PGE<sub>2</sub> and TNF- $\alpha$  ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). The PGE<sub>2</sub> kit is a competitive type immunoassay while the TNF- $\alpha$  kit is a direct capture assay. In each case, sample measurements were performed in duplicate and values were interpolated from standard curves. Optical density was determined using a PolarStar microplate reader (BMG Labtechnologies, Durham, NC, USA). For cytokine analysis, the RayBio Mouse Inflammation Antibody Array I was purchased from RayBiotech, Inc. (Norcross, GA, USA). According to manufacturer's instructions, the array membranes were incubated with blocking buffer followed by undiluted culture supernatants for 1.5 h. The membranes were washed and incubated with biotin-conjugated Abs for 1.5 h and HRP-conjugated streptavidin for 2 h. The membranes were next incubated in detection buffer and exposed to X-ray film. Finally, scans of the X-ray films were analyzed with Photoshop (Adobe) to determine spot density.

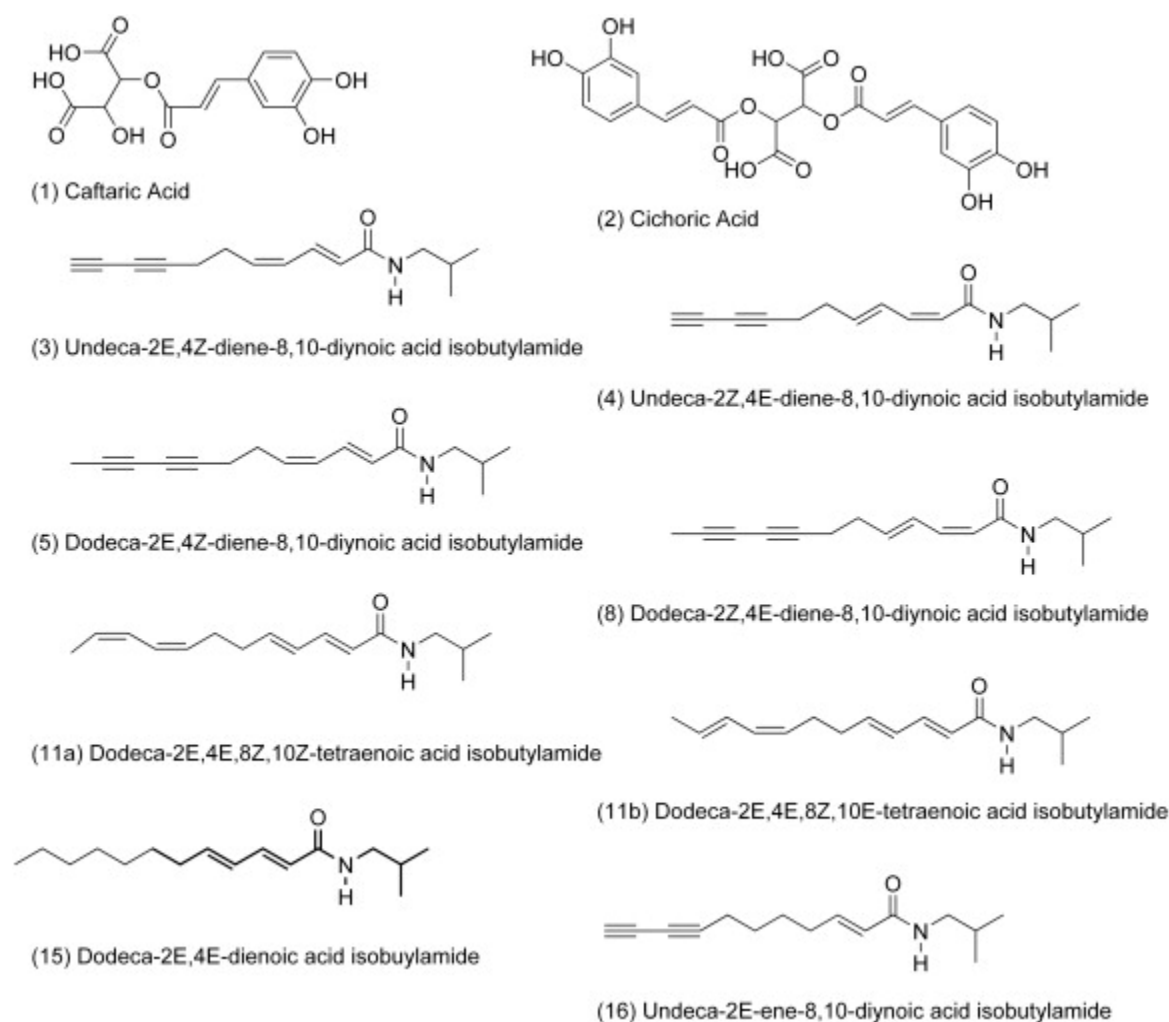
## 2.7. Statistical analysis

Significant differences between means were determined using unpaired Student's t-tests with 95% confidence intervals, as calculated with PRISM® software (Graphpad Software, San Diego, CA, USA). Correlation between PGE<sub>2</sub> and TNF- $\alpha$  concentration and levels of constituents (alkylamides, caftaric acid and cichoric acid) was evaluated using the Pearson test with SPSS software (IBM, Chicago, IL, USA).

# 3. Results

## 3.1. Effects of individual alkylamides on cytokine/chemokine secretion by RAW 264.7 macrophage-like cells

Fig. 1 shows a comparison of the structures of representative caffeic acid derivatives (1,2) and alkylamides (3–16) found in *Echinacea* species. Numbering is consistent with a previous publication<sup>[32]</sup>, and can be cross referenced to the numbering system used by Bauer<sup>[31]</sup> as indicated in the figure caption. All of the compounds in Fig. 1 were detected in the *E. purpurea* extracts investigated except alkylamide 16 (undeca-2E-ene-8,10-diynoic acid isobutylamide), which is a constituent of *E. angustifolia* that was tested here for activity in its purified form.



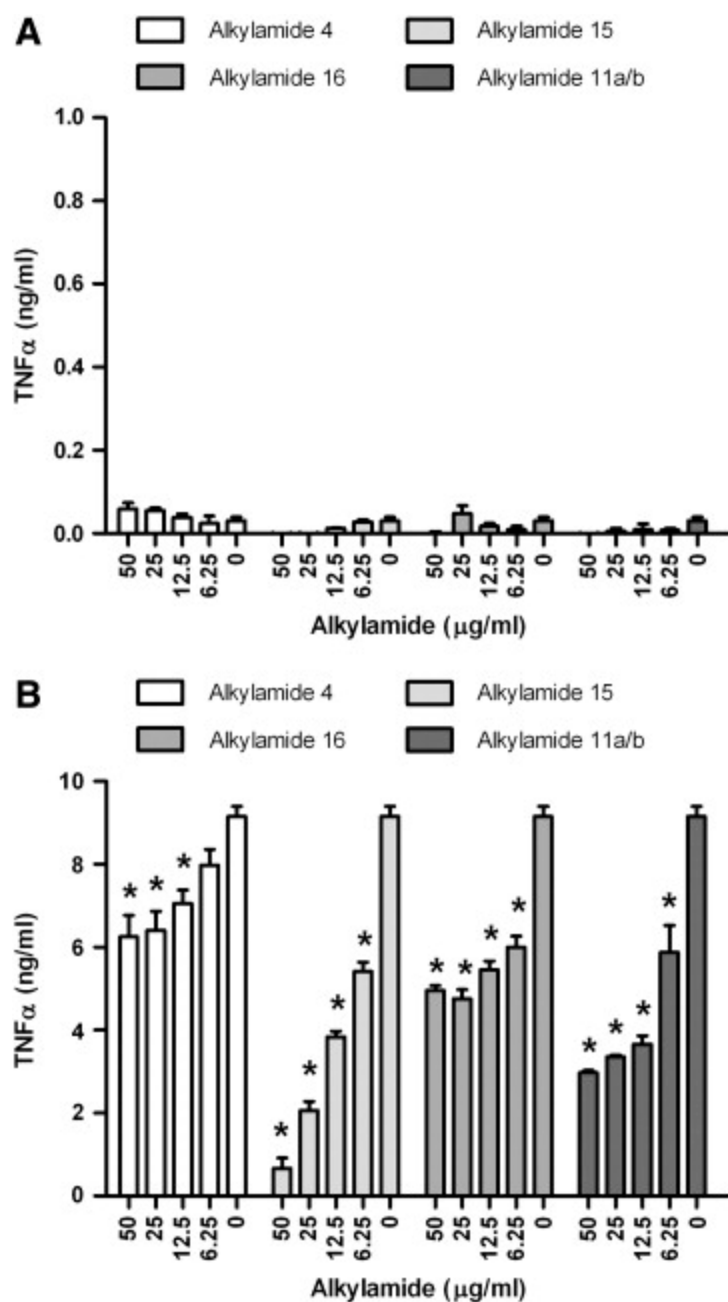
**Fig. 1.** Structures of caffeic acid derivatives (1 and 2) and alkylamides (3, 4, 5, 8, 11a, 11b, and 15) present in *Echinacea* species. All of these compounds except 16 are common constituents of *E. purpurea* and were detected in the extracts prepared for this study. Compound 16 is a common constituent of *Echinacea angustifolia* and was not detected in the *E. purpurea* extracts. However, its anti-inflammatory activity alone was investigated ( Fig. 2). The numbering system used here is consistent with a previous publication<sup>[32]</sup>. For cross-referencing, alkylamides 3, 4, 5, 11a, 11b, 15, and 16 in this paper correspond to alkylamides 1, 2, 3, 9, 8, 11, and 12,



respectively, by the Bauer numbering system<sup>[31]</sup>. Alkylamide 8 in the figure was not included in the original Bauer publication.

To evaluate the effects of alkylamide structure on inflammation, a set of four purified alkylamides was tested for their effects on the ability of RAW 264.7 macrophage-like cells to produce inflammatory products following infection with the influenza A virus strain PR/8/34. Included in this set were alkylamides 4 (undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide), a mixture of isomers 11a and 11b (11a/b, dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide), 15 (dodeca-2E,4E-dienoic acid isobutylamide), and 16 (undeca-2E-ene-8,10-diynoic acid isobutylamide). These alkylamides all contain the same isobutyl-substituted amide but vary in the length and or level of saturation of the alkyl chain (Fig. 1). The alkyl chains of alkylamides 15, 11a/b, 4, and 16 contain only single, two double, and two triple bonds, respectively.

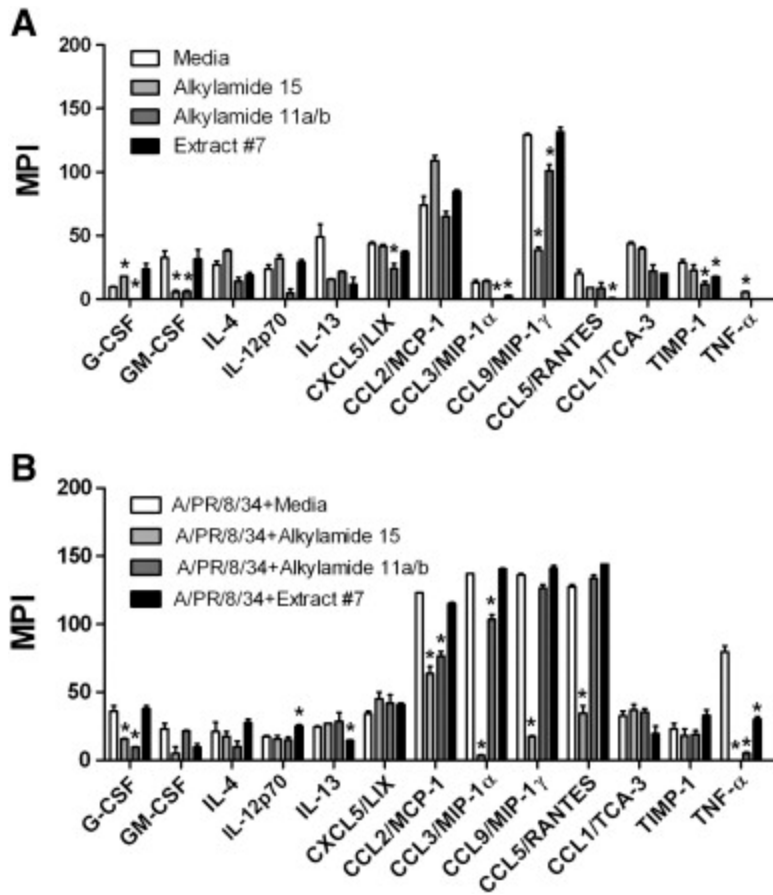
Initially, we investigated effects of these molecules on a key inflammatory mediator, TNF- $\alpha$ <sup>[27], [28],[29] and [30]</sup>. When tested with control cells, none of the alkylamides induced significant production of TNF- $\alpha$  (Fig. 2A,  $p < 0.05$ ). In contrast, as shown in Fig. 2B, we found that all of the alkylamides tested suppressed production of TNF- $\alpha$  in a dose dependent fashion from RAW 264.7 cells infected with influenza A strain PR/8/34 (Fig. 2B). Of the four alkylamides tested, alkylamide 4 and alkylamide 16 displayed the lowest levels of suppressive activity; alkylamides 11a/b displayed an intermediate level of suppression, while alkylamide 15 displayed a particularly pronounced suppressive effect on production of TNF- $\alpha$  (Fig. 2B). It is possible that the strong suppressive activity of alkylamide 15 arises from the level of saturation in its alkyl chain; the isomeric mixture of alkylamides 11a/b, which was less active than alkylamide 15, possesses two extra double bonds. The di-acetylene bonds (two triple bonds) in alkylamides 4 and 16 may be responsible for the low activity of these molecules.



**Fig. 2.** Production of TNF- $\alpha$  from alkylamide treated RAW 264.7 macrophage-like cells. Cells alone (A) or cells infected with influenza strain A/PR8/34 (B) (moi = 5) were treated with the indicated concentrations of alkylamides and supernatants collected after 24 h. In panel B, infections were initiated 30 min prior to the addition of the alkylamides. Values shown are means  $\pm$  SEM from two independent experiments. Each supernatant was assayed in duplicate by ELISA. Asterisks indicate significant differences (p < 0.05, t-test) from untreated cells. The isomeric alkylamides 11a and 11b were tested as a mixture.

To determine if the effects of these molecules are specific for TNF- $\alpha$ , or if they also affect production of other cytokines and chemokines, the two most potent alkylamides (15 and 11a/b)

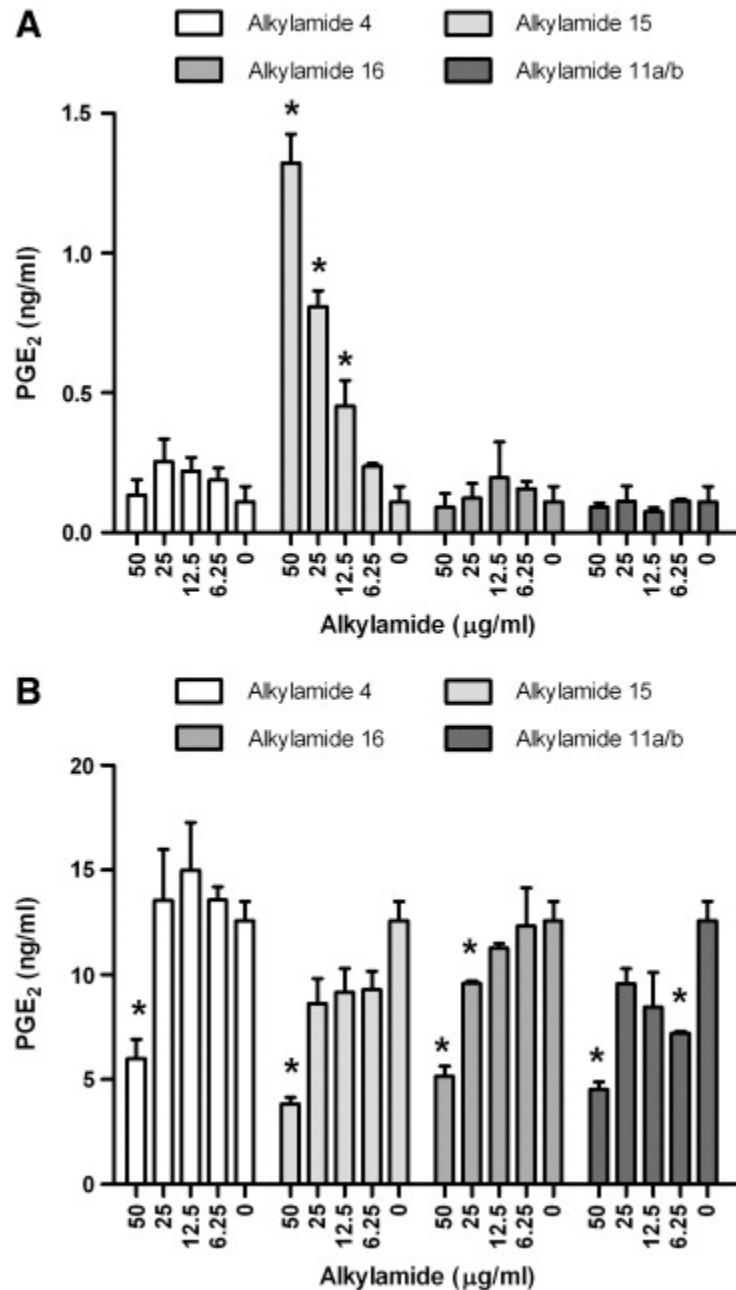
and a crude *E. purpurea* extract were tested for their effects on the production of multiple cytokines and chemokines using an antibody array. The array allows for the semi-quantitative identification of 40 different inflammatory proteins. Of these, 13 products were detected in supernatants from the control, alkylamide treated, raw extract treated (discussed later), and influenza A-infected RAW 264.7 cells ( Fig. 3). The other 27 products were not detected under any treatment condition. The control cells produced 10 products at low levels (G-CSF, GM-CSF, IL-4, IL12p70, IL-13, CXCL5/LIX, CCL3/MIP-1 $\alpha$ , CCL5/RANTES, CCL1/TCA-3, and TIMP-1), while two chemokines (CCL2/MCP-1 and CCL9/MIP-1 $\gamma$ ) were produced at higher levels ( Fig. 3A). Of the cytokines produced at low levels, alkylamide 15 caused significant enhancement of G-CSF and TNF- $\alpha$  ( $p < 0.05$ ) while inhibiting production of GM-CSF ( $p < 0.05$ ). The alkylamides 11a/b significantly inhibited production of G-CSF, GM-CSF, CCL5/LIX, CCL3/MIP-1 $\alpha$  and TIMP ( $p < 0.05$ ). Of the chemokines produced constitutively at high levels, neither alkylamide altered production of CCL2/MCP-1, while both alkylamides inhibited production of CCL3/MIP1- $\alpha$  ( $p < 0.05$ ). Infection of RAW 264.7 cells with influenza A strain PR/8/34 caused increased expression of G-CSF, CCL2/MCP-1, CCL3/MIP-1 $\alpha$ , CCL5/RANTES, and TNF- $\alpha$  ( Fig. 3B). For four of these molecules, G-CSF, CCL2/MCP-1, CCL3/MIP-1 $\alpha$ , and TNF- $\alpha$ , both alkylamide 15 and alkylamides 11a/b exerted significant suppressive effects ( $p < 0.05$ ). In addition, alkylamide 15 strongly inhibited production of CCL9/MIP-1 $\gamma$  and CCL5/RANTES while alkylamides 11a/b did not suppress production of these molecules. Taken together, these results show that alkylamides are capable of suppressing production of multiple cytokines and chemokines from influenza A-infected RAW 264.7 macrophages, but there are clear differences in effectiveness for the different alkylamides.



**Fig. 3.** Influence of alkylamides and a crude *E. purpurea* extract on the production of cytokines and chemokines by RAW 264.7 cells. Cells alone (A) or cells infected with influenza A strain PR/8/34 (B) were treated with media, the indicated alkylamides or extract #7 for 24 h. Infections were initiated 30 min prior to the addition of the alkylamides or the extract. Supernatants separate from those used in Fig. 2 were collected independently and a 1:4 dilution incubated with array membranes per manufacturer's instructions. Arrays were developed and spot intensity determined with Photoshop using digital images. Values shown are mean pixel intensities  $\pm$  SE from duplicate spots from a representative array. Asterisks indicate significant differences ( $p < 0.05$ , t-test) from untreated cells. The concentration of alkylamides was 25  $\mu$ M and a dilution of 85% ethanol (precipitated) extract was used to produce a final concentration of 22  $\mu$ M dodecatetraenoic acid isobutylamide (11a/b).

Production of inflammatory lipids is also an important component of influenza A mediated pathology [27], therefore, the alkylamides were tested for their effects on the production of PGE<sub>2</sub>. The results were somewhat different than those for TNF- $\alpha$ . When tested on their own, three of the four alkylamides did not affect production of PGE<sub>2</sub>, whereas alkylamide 15 induced significant levels of PGE<sub>2</sub> in culture supernatants (Fig. 4A). As shown in Fig. 4B, when tested with RAW 264.7 cells infected with strain PR/8/34, all the alkylamides inhibited production of

PGE<sub>2</sub> when used at a concentration of 50  $\mu$ M. In general, however, significant suppression of PGE<sub>2</sub> production was not seen with lower concentrations of the alkylamides.



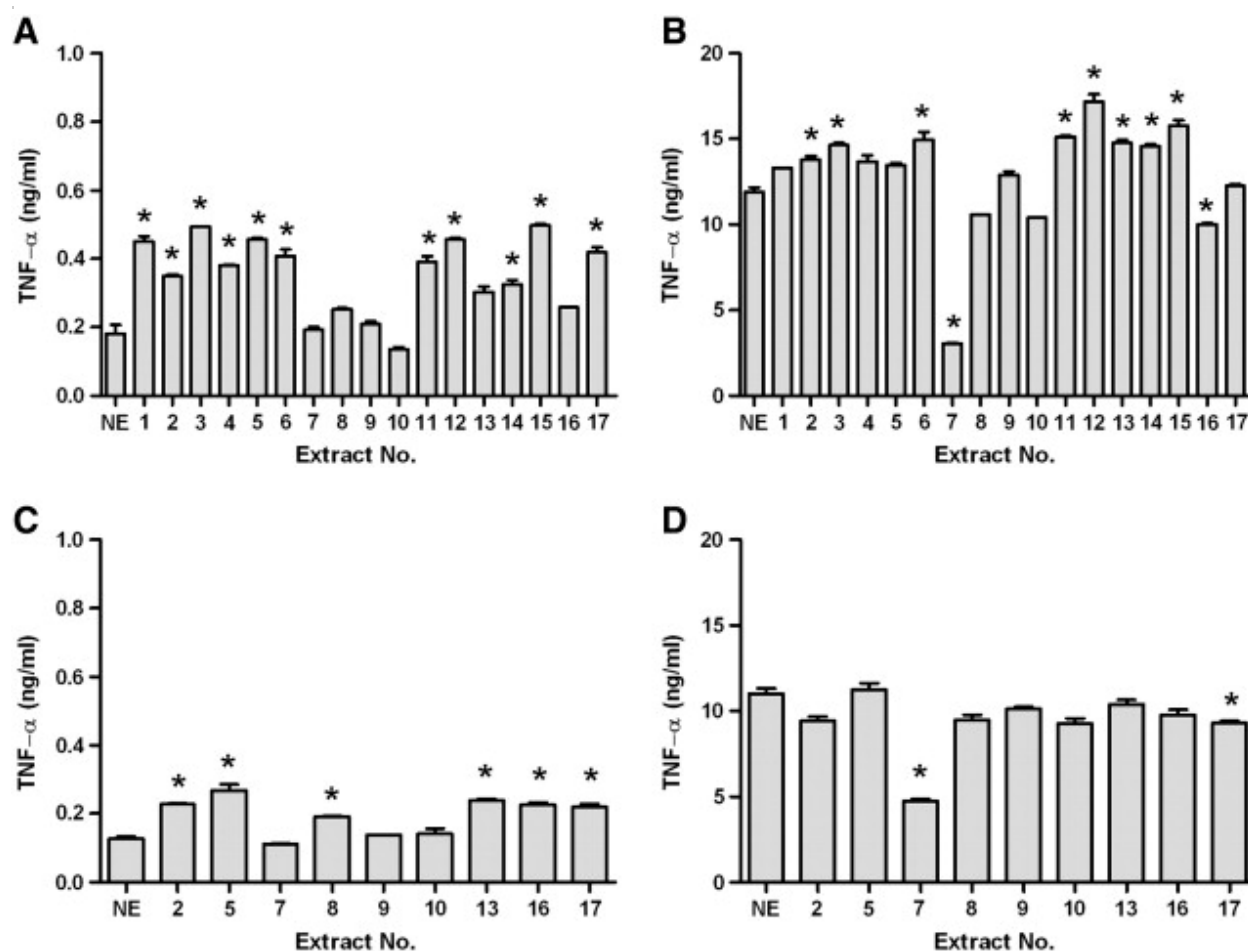
**Fig. 4.** Production of PGE<sub>2</sub> from alkylamide treated RAW 264.7 macrophage-like cells. Cells alone (A) or cells infected with influenza strain A/PR8/34 (B) (moi = 5) were treated with the indicated concentrations of alkylamides and supernatants collected after 24 h. In panel B, infections were initiated 30 min prior to the addition of the alkylamides. Values shown are means  $\pm$  SEM from three independent experiments. Each supernatant was assayed in duplicate by ELISA. Asterisks indicate significant differences ( $p < 0.05$ , t-test) from untreated cells. The isomeric alkylamides 11a and 11b were tested as a mixture.

### 3.2. Effects of complex *E. purpurea* extracts on production of cytokines, chemokines and PGE<sub>2</sub> by RAW 264.7 cells

One of the goals of these studies was to determine the role that alkylamides play in the anti-inflammatory activity of complex *E. purpurea* extracts. Toward this goal, seventeen crude extracts were prepared from dormant *E. purpurea* roots harvested from different locations across North Carolina ( Fig. 5). The extracts were tested for their ability to induce production of TNF- $\alpha$  ( Fig. 6A), and inhibit influenza A-induced TNF- $\alpha$  production ( Fig. 6B). A number of the extracts did not affect levels of constitutive expression, while several actually significantly ( $p < 0.05$ ) enhanced production of TNF- $\alpha$ , with levels rising more than twofold vs. control cells. As expected, infecting RAW 264.7 cells with influenza A strain PR/8/34 led to high levels of TNF- $\alpha$  (note the change in the scale of the y-axis) and again a range of effects was observed for the extracts ( Fig 6B). Several enhanced production up to 40%, several exerted no effect, while only extract 7 exerted a strong suppressive effect ( $p < 0.05$ ). A weak but significant suppressive effect was noted in these experiments for extract 16, but this result was not confirmed in subsequent experiments (see below). In general, the extracts that stimulated production of TNF- $\alpha$  when used on their own also stimulated production from infected cells. Conversely, those extracts that displayed minimal activity when tested on their own also displayed minimal activity with infected cells.



**Fig. 5.** Map of NC indicating the counties where the *E. purpurea* root samples were collected.



**Fig. 6.** Production of TNF- $\alpha$  from crude *E. purpurea* extract treated RAW 264.7 macrophage-like cells. Each ethanol extract was added to a 1 mL culture of either cells alone (A and C) or cells infected with influenza strain A/PR8/34 (B and D) (moi = 5) and supernatants collected after 24 h. In panels B and D, infections were initiated 30 min prior to the addition of the extracts. Panels A and B show results from treatments with 6.7  $\mu$ L of 75% ethanol extracts while in panels C and D 5.8  $\mu$ L of 85% ethanol (precipitated) extracts were added. Assay concentrations of dodecatetraenoic acid isobutylamide (11a/b) were in the range of 5–20  $\mu$ M (Table 1). No extract (NE) control cultures included identical amounts of the appropriate ethanol concentrations. Values shown are means  $\pm$  SEM from two independent experiments. Each supernatant was assayed in duplicate by ELISA. Asterisks indicate significant differences ( $p < 0.05$ , t-test) from untreated cells.

In recent literature, it has been reported that much of the ability of *Echinacea* extracts to stimulate production of inflammatory cytokines from macrophages may be caused by the presence of lipopolysaccharides or lipoproteins<sup>[18] and [19]</sup>. With this in mind, the extracts were precipitated with ethanol, which can remove these products and which increased the final ethanol concentration to 85%. A subset of these precipitated extracts was then tested for their effects on

constitutive and influenza A-induced production of TNF- $\alpha$ . As shown in Fig. 6C, when a subset of the precipitated extracts were added to control cells, the magnitude of the stimulatory effect was reduced, however, several still produced significant ( $p < 0.05$ ) increases in TNF- $\alpha$  production. A more pronounced effect was noted when influenza A-infected cells were treated with the precipitated extracts. None of these extracts induced significant increases in TNF- $\alpha$  production (Fig. 6D) while two of the extracts (7 and 17) did significantly ( $p < 0.05$ ) inhibit production of TNF- $\alpha$ . The suppressive effect noted in Fig. 6B for extract 16 was not confirmed in these experiments. It is possible that precipitation with ethanol diluted the extract, thereby reducing its all ready weak suppressive activity.

As shown in Fig. 6D, one extract (7) strongly suppressed production of TNF- $\alpha$  in a manner similar to the purified alkylamides (Fig. 2); therefore, we investigated the effects of this extract on the production of additional cytokines and chemokines. As shown in Fig. 3A, with control cells, the extract significantly ( $p < 0.05$ ) suppressed production of CCL3/MIP-1 $\alpha$ , CCL5/RANTES, and TIMP-1. With infected cells, the extract enhanced production of IL-12p70 while inhibiting production of TNF- $\alpha$  and IL-13 ( $p < 0.05$ ). Unlike the purified alkylamides, however, the crude extract did not affect the influenza A-induced production of G-CSF, CCL2/MCP-1, CCL3/MIP-1 $\alpha$ , CCL9/MIP-1 $\gamma$ , or CCL5/RANTES (Fig. 3B).

The crude, 75% ethanol extracts were also tested for their effects on production of PGE<sub>2</sub> (Fig. 7). With control cells, only one extract (12) caused significant stimulation of PGE<sub>2</sub> production (Fig. 7A). In contrast, when tested with influenza A-infected cells, several of the extracts strongly enhanced PGE<sub>2</sub> production while one (7) significantly inhibited production (Fig. 7B). To determine whether the stimulatory effects of the extracts could be attributed to bacterial products that may have been present, the extracts were retested after a single round of ethanol precipitation (Fig. 7C and D). With control cells, several of the precipitated extracts did cause significant ( $p < 0.05$ ) but small increases in production of PGE<sub>2</sub> (note the scale of the y-axis). More dramatically, when the precipitated extracts were tested with infected cells, none was found to enhance PGE<sub>2</sub> production and several were found to significantly ( $p < 0.05$ ) inhibit production. Again we noted that extract 7 produced the strongest suppressive effect.



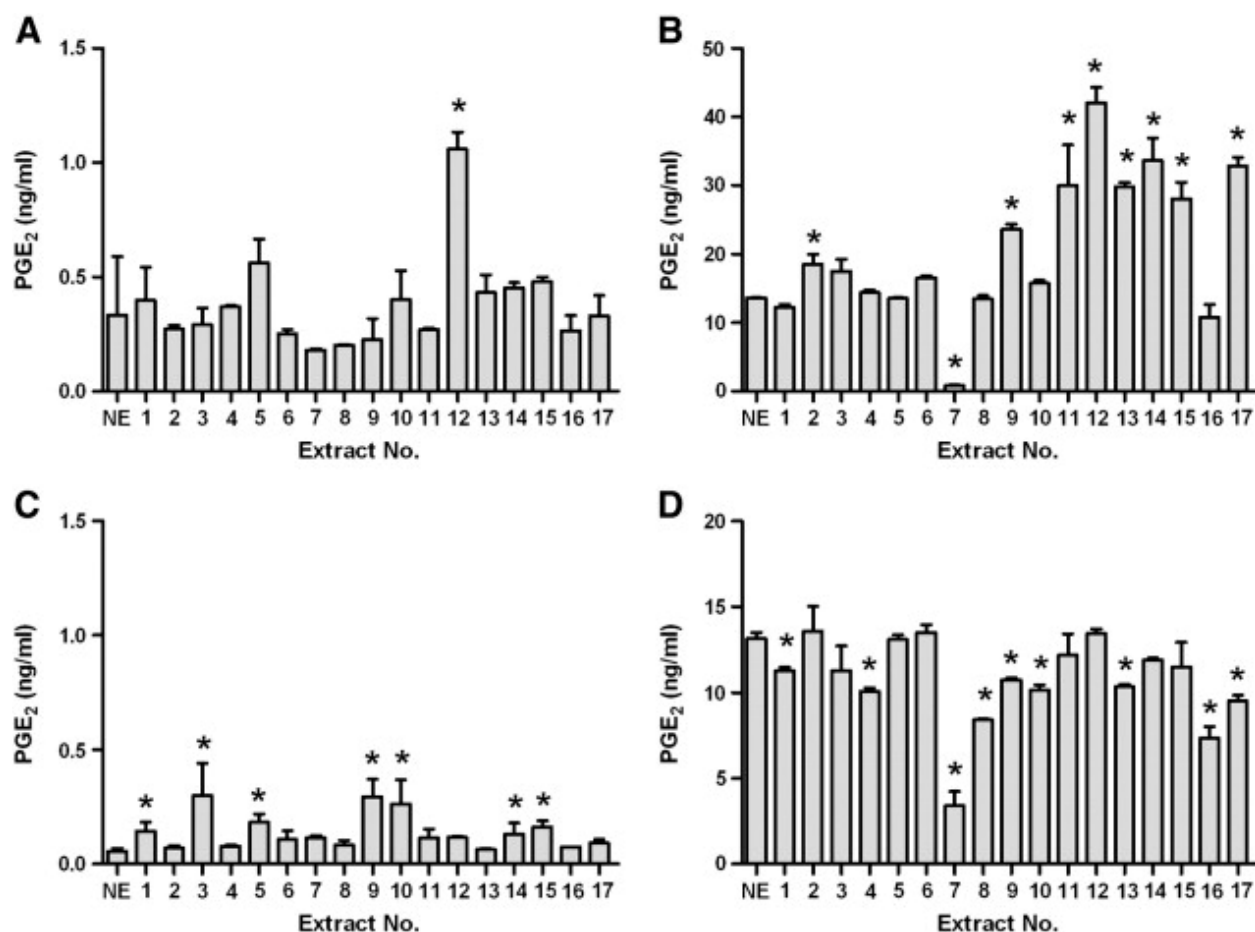


Fig. 7. Production of PGE<sub>2</sub> by RAW 264.7 macrophage-like cells treated with crude *E. purpurea* extracts. Each ethanol extract was added to a culture of either cells alone (A and C) or cells infected with influenza strain A/PR8/34 (B and D). Panels A and B show results from treatments with 6.7 µL of 75% ethanol extracts while in panels C and D 5.8 µL of 85% ethanol (precipitated) extracts were added. Conditions were identical to those listed for Fig. 6, except that ELISA was conducted for PGE<sub>2</sub> instead of TNF-α. Asterisks indicate significant differences ( $p < 0.05$ , t-test) from untreated cells.

### 3.3. Alkylamide and caffeic acid derivative content of crude *E. purpurea* extracts and relation to activity

Quantitative analysis of several major constituents (alkylamides 11a/b, caftaric acid and cichoric acid) was conducted to determine whether their concentration would correlate with the observed anti-inflammatory activity of the extracts. The assay concentration of alkylamide 11a/b in the precipitated extracts was observed to vary from 1.1 µg/mL to 5.4 µg/mL, a range of 5-fold (Table 1). However, the concentration of this constituent in the precipitated extracts was only moderately correlated with concentration of PGE<sub>2</sub> ( $r = -0.496$ ,  $p = 0.04$ , Pearson) and TNF-α ( $r = -0.608$ ,  $p = 0.03$ ) in the cell media. Furthermore, there was no significant correlation ( $p < 0.05$ ) between concentration of PGE<sub>2</sub> or TNF-α in the cell media and concentration of

caftaric or cichoric acid in the precipitated extracts (for caftaric acid and PGE<sub>2</sub>,  $r = -0.225$ ,  $p = 0.4$ ; for caftaric acid and TNF- $\alpha$ ,  $r = -0.173$ ,  $p = 0.6$ , for cichoric acid and PGE<sub>2</sub>,  $r = -0.025$ ,  $p = 0.9$ ; for cichoric acid and TNF- $\alpha$ ,  $r = -0.133$ ,  $p = 0.7$ ).

*E. purpurea* contains at least 16 different structurally diverse alkylamides<sup>[33]</sup>. For this study, only the most abundant of these, dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide (11a/b) was quantified. It was of interest, however, to evaluate whether the presence of some of the other minor alkylamides would correlate with observed anti-inflammatory activity of the extracts. To accomplish this goal, correlation between HPLC peak area and concentration of PGE<sub>2</sub> or TNF- $\alpha$  in the cell media was evaluated for each of the major alkylamides detected in the extract (compounds 3, 4, 5, 8, 11a/b, and 15 in Fig. 1). A moderate negative correlation was observed between the concentration of both PGE<sub>2</sub> and TNF- $\alpha$  and the peak area of alkylamides 11a/b. This would be expected given the aforementioned correlation between concentration of alkylamide 11a/b and levels of PGE<sub>2</sub> and TNF- $\alpha$ . No correlation between PGE<sub>2</sub> or TNF- $\alpha$  concentration and peak area for any of the other alkylamides (3, 4, 5, 8, 15) was observed.

#### 4. Discussion

Influenza A is a virus that represents a continuous threat to human and animal health. Thousands of people die each year from the relatively mild strains of seasonal influenza A and, occasionally, highly pathogenic pandemic strains emerge as more serious threats. The virus' genetic information is in a continuous state of flux, making the continuous development of new vaccines a necessity. The genetic malleability of this virus also means that resistance to anti-viral agents develops with predictability and that constant development of new anti-viral compounds is also a necessity. Numerous investigations have shown that it is not the growth of the virus per se that causes many of the symptoms and pathology of the disease. Instead, many of the symptoms and pathology stem from the macrophage-dependent storm of inflammatory cytokines, chemokines, and inflammatory lipids that are triggered by the virus. Therefore, we investigated the effects of purified alkylamides and *Echinacea* extracts on the ability of influenza A strain PR/8/34 to induce the production of inflammatory products from RAW 264.7 macrophage-like cells.

All of the alkylamides tested suppressed production of TNF- $\alpha$  from virus infected cells. These data, in combination with previous results showing inhibition of TNF- $\alpha$  and other cytokines from LPS treated cells<sup>[23]</sup> and<sup>[24]</sup>, suggest that alkylamides can mediate a ligand-independent effect on the production of TNF- $\alpha$ . Two of the alkylamides also displayed inhibitory activity against a number of additional cytokines and chemokines, including; G-CSF, GM-CSF, CCL2/MCP-1, CCL3/MIP-1 $\alpha$ , CCL9/MIP-1 $\gamma$  and CCL5/RANTES. The mechanism underlying these effects is not known. A trypan blue exclusion assay failed to reveal evidence of alkylamide-induced cell death (data not shown) and, as shown in Fig. 3, the production of several cytokines and chemokines was unaffected by the alkylamides. Together, these findings argue against any broad toxic or inhibitory effect of the alkylamides on cellular metabolism. The finding that several

cytokines and chemokines are produced equally in the absence or presence of the alkylamides also argues against the alkylamides exerting a strong suppressive effect on virus replication. The host response against influenza A is triggered by viral RNA interacting with a number of pattern recognition receptors including TLR-3, -7, -9, and RIG-I (reviewed in [34]) and strong inhibition of viral RNA production would likely cause broad, uniform inhibition of the host response. More likely, the effect of the alkylamides on production of TNF- $\alpha$  and other cytokines and chemokines stems from effects on specific transcription factors. An inhibitory effect on NF- $\kappa$ B might explain the effects of alkylamide 15 (dodeca-2E,4E-dienoic acid isobutylamide), for example, since expression of TNF- $\alpha$  [35], MCP-1 [35], and G-CSF [36] are dependent on this transcription factor. An alkylamide has also been implicated in activation of the orphan receptor PPAR $\gamma$  [37] which, along with the orphan receptors, is known to control the activity of many genes. On the other hand, arguing against a suppressive effect on transcription factors is the finding that *Echinacea* extracts have been shown to enhance the production of multiple transcription factors [38]. It is also unclear if alkylamide binding to the cannabinoid type 2 (CB<sub>2</sub>) receptor is responsible for these effects. Previously, alkylamides 11a/b and 15 have both been shown to bind the CB<sub>2</sub> receptor [24] and [26]. However, these alkylamides displayed distinct effects on cytokine and chemokine production, which suggests that distinct receptors may be involved. Clearly, additional experiments will be necessary to define the effects of the alkylamides on cytokine and chemokine expression.

The results of our experiments also revealed inhibition of PGE<sub>2</sub> production by the alkylamides. This effect was seen primarily at the 50  $\mu$ M concentration, and the dilutions we tested did not produce readily interpretable dose response curves. In addition, we found that alkylamide 15 stimulated production of PGE<sub>2</sub> when used with control cells but inhibited production of PGE<sub>2</sub> by virus-exposed cells. It is possible, therefore, that the alkylamides are exerting multiple, complex effects on the biochemical pathways responsible for production of PGE<sub>2</sub>. Alkylamide 15, for example, might be stimulating constitutive, COX-1-dependent PGE<sub>2</sub> production, while inhibiting influenza A-induced, COX-2-dependent production of PGE<sub>2</sub>. Stimulation of constitutive PGE<sub>2</sub> production might arise from kinase activation and the phosphorylation of cPLA<sub>2</sub>, while inhibition of influenza A-dependent production of PGE<sub>2</sub> might occur because expression of COX-2 is blocked. De novo transcription of COX-2 is necessary for PGE<sub>2</sub> production in RAW 264.7 cells [39]. Alternatively, the alkylamides could be mediating their effects on PGE<sub>2</sub> through the induction of nitric oxide [40]. Nitric oxide has been shown to enhance COX-1-dependent, constitutive production of PGE<sub>2</sub> and at the same time inhibit COX-2-dependent, inducible production of PGE<sub>2</sub> [41]. Again, additional experiments will be necessary to resolve the effects of the alkylamides on production of PGE<sub>2</sub>.

In contrast to the effects of the purified alkylamides, the *E. purpurea* extracts tested did not generally display suppressive activities. We did observe a moderate correlation between alkylamide 11a/b concentration in the precipitated extracts and concentration of TNF- $\alpha$  ( $r = -0.608$ ) and PGE<sub>2</sub> in the cell media ( $r = -0.496$ ). These values are indicative of a trend

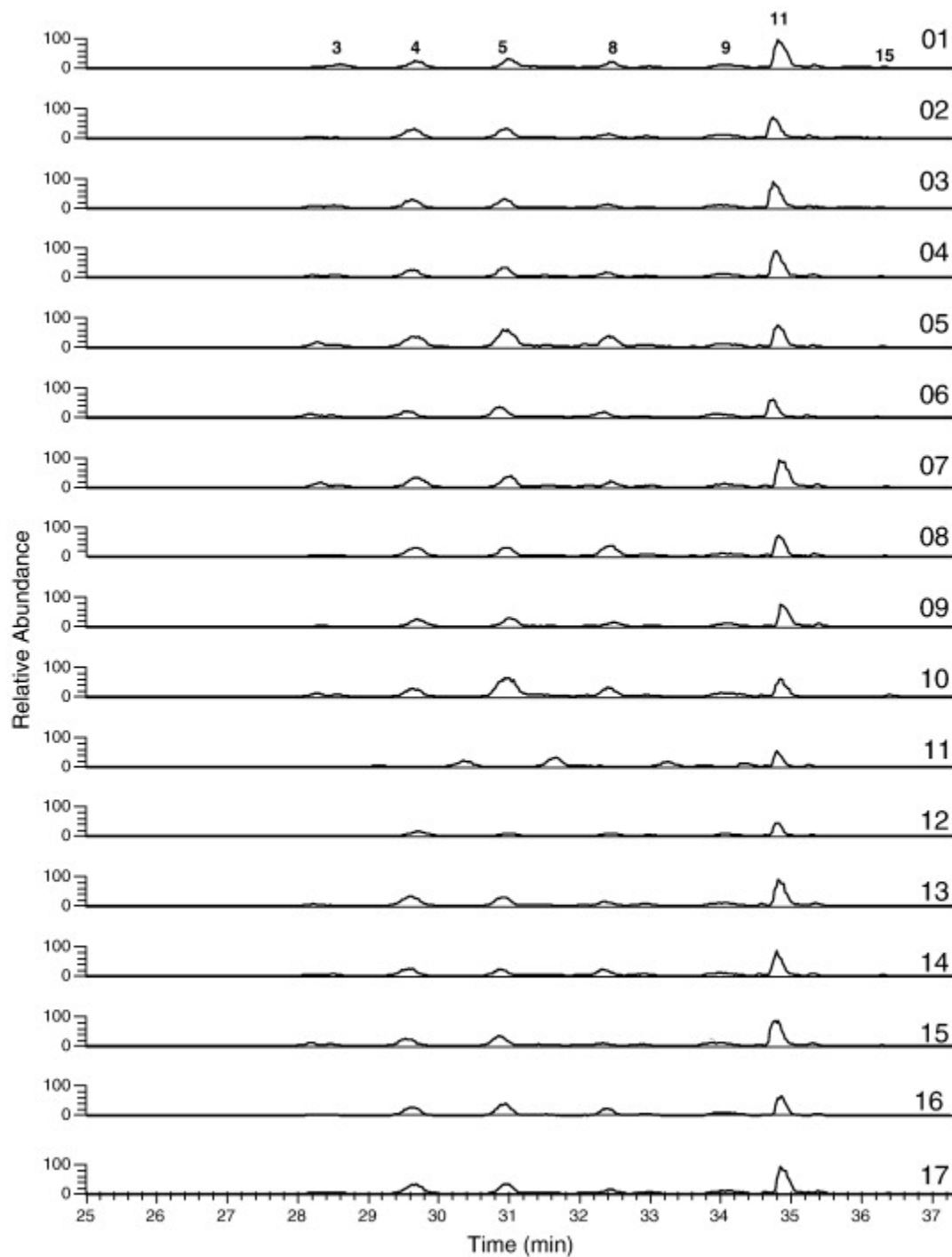
towards a suppressive effect of alkylamide 11a/b on secretion of TNF- $\alpha$  and PGE<sub>2</sub> by the RAW 264.7 cells. However, only extract 7 strongly inhibited production of both TNF- $\alpha$  and PGE<sub>2</sub>. This extract also partially suppressed the production of TNF- $\alpha$  and IL-13 from influenza infected cells. Why the extracts did not display the anti-inflammatory activity displayed by the purified alkylamides is not clear. One explanation for this observation would be that levels of alkylamides in the extracts were not sufficient to accomplish the effects seen with the isolated compounds. The concentrations of alkylamide 11a/b in the extracts (up to 5  $\mu\text{g/mL}$ ), however, were on the same order as the lowest concentration tested for this compound alone (6.25  $\mu\text{g/mL}$ ), and alkylamide 11a/b was still quite active at 6.25  $\mu\text{g/mL}$  (Fig. 2). Furthermore, the extracts were shown to contain a number of additional alkylamides (Fig. 7); therefore, the total alkylamide concentration would certainly have been well within the active range. Finally, inhibitory activity was observed for several extracts that contained similar alkylamide levels to other inactive extracts (Fig. 7). All of these factors suggest that low alkylamide concentration could not account for the observed lack of activity of some of the extracts.

Another possible explanation for the relative inactivity in the extracts is the presence of stimulatory molecules that work in opposition to the effects of inhibitory alkylamides. Several studies have shown that *Echinacea* extracts can contain plant polysaccharides or glycoproteins<sup>[9]</sup> or products of (possibly endophytic) bacteria, such as lipopolysaccharide or lipoprotein<sup>[18]</sup> and<sup>[19]</sup>, which stimulate macrophage activity. In agreement, our ethanol precipitation experiments (which should decrease the concentration of these stimulatory molecules) did reduce the stimulatory activity of the extracts used in these experiments. In fact, suppressive effects were revealed upon precipitation for some of some extracts that showed no suppressive activity prior to precipitation. We did not test whether ethanol precipitation can routinely and uniformly reduce the macrophage-stimulatory activity in *Echinacea* extracts or in plant extracts in general.

We were able to rule out lipopolysaccharide (LPS) as the activating constituent in the extracts. As shown by the limulus amoebocyte lysate (LAL) assay, extracts contained less than 0.25 EU/mL of LPS. This corresponds to an assay concentration of less than 0.1 pg/mL, approximately 10,000 times lower than the 1 ng/mL LPS concentration limit required to activate macrophages in our assay. Furthermore, addition of polymixin B, a compound that abrogates the effects of LPS<sup>[42]</sup>, did not restore activity of the inactive extracts (data not shown). It is still possible that bacterial-derived lipoproteins, which are not detected with the LAL assay, are responsible for the stimulatory activity in the extracts. These, however, should also precipitate at high ethanol percentages.

Alternatively, it is possible that the extracts did not act as did the purified alkylamides, because the mixture of alkylamides present in the extracts (Fig. 8) does not behave additively, or synergistically, but instead delivers contradictory signals to the macrophage. Several alkylamides have been shown to bind to the CB<sub>1</sub> and CB<sub>2</sub> receptors<sup>[24]</sup> and<sup>[26]</sup>, and Gertsch et al.<sup>[23]</sup> have reported that certain alkylamides can induce production of TNF- $\alpha$  mRNA (but not protein) in a

CB<sub>2</sub> receptor-dependent fashion. Alkylamides have also been shown to inhibit the LPS-induced expression of inflammatory cytokines in a CB<sub>2</sub>-independent manner<sup>[24]</sup>, and to modulate several signal transduction and second messenger systems including cAMP, Ca<sup>++</sup>, and MAP kinases<sup>[23]</sup>. It is possible, therefore, that a complex mixture with multiple alkylamides generates contradictory signals into the cell, resulting in no net effect.



**Fig. 8.** Chromatograms comparing alkylamide profiles in different *E. purpurea* root extract samples. Analysis was conducted via HPLC/ESI-MS in the positive ion mode using a previously published method [33]. Chromatograms are base peak chromatograms, which plot the intensity of the most abundant ion vs. time. Note the remarkable similarity in alkylamide profile between the samples, for example sample 2 (inactive) has an extremely similar profile to sample 7 (highly active). There was no correlation between peak area of any of the alkylamides and suppression of PGE<sub>2</sub> secretion (evaluated by Pearson).

Regardless of the reason, these data indicate that the effects of crude ethanolic *E. purpurea* extracts on the ability of influenza A to stimulate mediator production from macrophages in vitro can vary significantly depending on the plant material used in their preparation. In agreement with previous reports [20],[21] and [23], we show that some crude ethanolic extracts of *E. purpurea* do, indeed, possess anti-inflammatory activity in vitro, and that alkylamides alone possess this activity as well. However, our results suggest that the widely held belief that alkylamide rich ethanolic extracts of *E. purpurea* should have anti-inflammatory activity, while polysaccharide rich aqueous extracts should stimulate immune response, may be an oversimplification. With our in vitro model, we show that even some alkylamide-containing extracts with relatively high ethanol content (75%) can stimulate cytokine production by virus-exposed macrophage-like cells in vitro, and that even increasing ethanol percentage to 85% does not restore anti-inflammatory activity for the stimulatory extracts. One important caveat when considering the relevance of these findings is that while the RAW 264.7 cell model is typically very predictive of in vivo effects [39], it does not take into account how the constituents of the extracts are absorbed or metabolized in the human body. If the extracts investigated in this study do contain stimulatory constituents that mask the anti-inflammatory activity of alkylamides, these same extracts could still display anti-inflammatory in vivo if the stimulatory molecules are not readily absorbed.

Ultimately, given the findings from our laboratories and others that show anti-inflammatory activity of alkylamides, extracts with high alkylamide concentration would be expected to have anti-inflammatory activity. Further studies are needed to investigate the role of constituents other than alkylamides in the activity of *E. purpurea* extracts before the observed variability in the anti-inflammatory activity of these extracts in vitro can be explained. Such studies are relevant to the quality control of *E. purpurea* extracts used as dietary supplements or for in vivo studies. When the issue of other masking constituents present in *E. purpurea* extracts is resolved, the levels of alkylamides may indeed serve as a predictive biomarker for anti-inflammatory activity.

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